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(54) Title: PREPARATION OF UNASSEMBLED PILUS SUBUNITS AND VACCINES CONTAINING THEM

(57) Abstract

A method for obtaining unassembled bacterial pilus subunits from specified bacteria is disclosed and claimed. Unassembled pilin thus obtained are useful for preparing vaccines which elicit enhanced cross-reactive antibodies with heterologous bacteria, and protect animals from infection.

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"PREPARATION OF UNASSEMBLED PILUS SUBUNITS AND VACCINES CONTAINING THEM."

Field of the Invention.

The present invention relates to a unique method for obtaining unassembled bacterial pilus subunits suitable for use in preparing a vaccine, as well as vaccines containing unassembled pilin and which exhibit enhanced cross-reactivity with heterologous bacteria and protect animals from infection.

Description of the Prior Art.

Reference is had to my prior U. S. Patent No. 4,702,911, the disclosure of which is hereby incorporated by reference. Described and claimed in that prior '911 patent is a unique method for obtaining bacterial pili subunits suitable for use in preparing a vaccine. While the method described and claimed in that prior patent is still quite suitable, further research has resulted in the discovery of an improved method for obtaining not only such pili subunits, but also for inhibiting the formation of assembled pili and for utilizing unassembled pilin in the preparation of vaccines. It has also been determined that by formulating vaccines from mixtures of unassembled pilin from a plurality of bacterial strains, a vaccine of enhanced cross-reactivity with heterologous bacteria is obtained.

As set forth in my prior '911 patent, pilus subunits have normally been prepared by dissaggregating pili. However, it is known that certain Neisseria gonorrhoeae isolates synthesize unassembled pili. It is believed that other genera of bacteria will be discovered to do this as well. This is important because nonpiliated bacteria are usually more amenable to growing in liquid culture than are the piliated forms, and most vaccine production facilities are already equipped to handle fluids quite efficiently. With particular regard to the use of unassembled pilin, it is known that a normally

occurring variant of g n coccus has b en shown by Swanson, et al., to have at least three pilus negative gonococcal phenotypes. Two of these phenotypes do not synthesize pilin, while the third synthesizes pilin but does not assemble it. See Swanson, J., O. Barrera and D. Corwin. 1985. P⁻ Gonococci Are Not All Equal. THE PATHOGENIC NEISSERIA, edited by G. K. Schoolnik, Am. Soc. Microbiol., Washington, D.C., pp. 281-287. Another article by Stephens, et al., indicates that subinhibitory concentrations of tetracycline and penicillin antibiotics allow synthesis of the pilin to proceed, but prevents its assembly into pili. See Stephens, D.S., J.W. Krebs, and C.A. McGee. 1984. Loss of Pili and Decreased Attachment to Human Cells by Neisseria meningitidis and Neisseria gonorrhoeae exposed to Subinhibitory Concentrations of Antibiotics. INFECT. IMMUN. 46:507-513.

Thus, notwithstanding my prior '911 patent, the primary difficulties associated with producing a pili subunit vaccine still basically involve determining a commercially-acceptable procedure for deriving the pili subunits so that a subunit vaccine can be readily prepared in accord with accepted manufacturing processes. By virtue of the present invention, those primary difficulties are substantially alleviated, for I have not only developed an improved method for obtaining unassembled pilus subunits, but also have developed a method utilizing unassembled pilin from bacteria capable of producing or modified to produce unassembled pilin, and utilizing those products have developed an improved vaccine having enhanced cross-reactivity with heterologous bacteria.

SUMMARY OF THE INVENTION

The present invention relates, first, to a unique method for obtaining unassembled bacterial pilus subunits directly from bacteria. As a result of my work to date, it seems clear that unassembled bacterial pilus subunits can be obtained from and vaccines can be developed against, virtually any piliated pathogenic bacteria, even those that are incapable of completing the assembly of the pilus. As is set forth in greater detail hereinafter, unassembled pilin have been obtained from Moraxella bovis, and vaccines prepared from the M. bovis unassembled pilin have demonstrated enhanced efficacy. Pili from M. bovis belong to the NMePhe pilus group, and this class of pili is found not only on M. bovis, but also on Neisseria gonorrhoeae, N. meningitidis, M. nonliquefaciens, M. catarrhalis, Vibrio sp., Pseudomonas aeruginosa, and Bacteriodes nodosus. Thus, the method described and claimed herein, as well as vaccines prepared therefrom, are specifically intended to fall within the scope of this invention. It is further believed that the method and vaccine of this invention would also be applicable for each of the pathogenic bacterium set forth at column 3 of my prior '911 patent.

Two basic methods for obtaining unassembled pilin in accord with the scope of this invention have been determined and are set forth in greater detail hereinafter. Both methods involve harvesting the bacteria substantially in accord with the disclosure of my prior patent or other known techniques. In the case of bacteria possessing intact pili, unassembled pilin may be obtained by first subjecting the pili to ultrasonication to dissociate the pili into pilin. This may normally be accomplished in a period of from 2 to 5 minutes using a sonication power of from about 35 to about 60 watts. The sonicated pilin are then dialysed against distilled water at a pH of from about 11.5 to

about 12.5 for a period of from 24 to 72 hours. The dialysed material is then adjusted to substantial neutral pH.

Alternatively, unassembled pilin may be obtained directly from bacteria which synthesize pilin but are incapable of assembling it. As set forth in the article by Stephens, above, subjecting bacteria to subinhibitory concentrations of antibiotic penicillin will allow synthesis of the pilin to proceed, but will prevent its assembly into pili, also resulting in a source of unassembled pilin for use in preparing the vaccine of this invention.

Once the unassembled pilin has been obtained, it may be preserved, stored or lyophylized for subsequent use in vaccine formulation, or one may proceed directly to vaccine formulation. In any event, of course, the concentration of the unassembled pilin would be adjusted as required for vaccine preparation. Vaccines formulated with unassembled pilin would normally include an adjuvant, but the use of an adjuvant is not required within the scope of this invention. In similar fashion, vaccines utilizing the unassembled pilin of this invention might also include preservatives and stabilizers. Furthermore, it has been found desirable to include unassembled pilin from more than one bacterial strain in the vaccine.

More specific details of the method of this invention will be set forth in the examples presented hereinafter. The invention accordingly comprises the several steps and the relation of one or more of such steps with respect to each of the others which will be exemplified in the method hereinafter disclosed, and the scope of the invention will be indicated in the claims.

DETAILED DISCUSSION

The present invention relates to a method for obtaining unassembled pilus subunits as well as vaccines containing unassembled pilin which elicit antibodies with enhanced cross-reactivity with heterologous bacteria, and protect animals from infection. The method of this invention has been demonstrated to be suitable for use in obtaining unassembled pilin from a variety of pathogenic bacteria, and detailed studies have been conducted with regard to M. bovis including a particularly effective vaccine prepared using aggregates of unassembled pilin from different isolates of M. bovis. As used herein, the term "oligomer" is synonymous with "unassembled pilus subunits" and with "unassembled pilin."

The following examples, then, are set forth in order to describe more fully the method and vaccine of the present invention.

EXAMPLE I

Moraxella bovis isolates FLA64(6), EPP63, MED72(23R), and NDL67(S-57R), were obtained from the National Animal Disease Center, AMES, Iowa. The MISS isolate was supplied by the Mississippi Diagnostic Laboratory, Jackson, Mississippi, and the IM427 was isolated locally from a Holstein cow in Hillsborough County, Florida.

Pili were prepared from the M. bovis as follows. M. bovis, grown for 24 hours on Mueller-Hinton agar was scraped into 10mM Tris-HCl buffer adjusted to pH 7.0 and blended for 2 minutes to shear the pili from the bacteria. After centrifugation, the supernatant was retained and ammonium sulfate was added to 20% saturation. After incubation for 1 to 2 hours in an ice bath, the solution was centrifuged for 2 hours. The pellet was retained and resuspended in Tris-HCl buffer. After resuspension, the solution was centrifuged for 2

hours, and the supernatant c ntaining the pili was saved.

The pili from the EPP63 strain were dissociated by ultrasonication for 2 minutes at a setting of 35 watts using a 0.4cm diameter probe, followed by dialysis for 24 to 48 hours against distilled water adjusted to pH 11.5. Oligomers from the other strains were prepared by ultrasonication for 2 minutes at 60 watts by placing the pili preparation in a glass test tube within another vessel and positioning a 2.0cm diameter probe in the outer vessel. This was followed by dialysis against water adjusted to pH 12.5. Conversion to oligomers was confirmed and the oligomers were dialyzed against 10mM, pH 7.0 Tris-HCl prior to formulation as a vaccine.

Vaccines were prepared in three fashions. A first vaccine was prepared with intact pili in a Freund's adjuvant. A second vaccine was prepared utilizing intact pili in aluminum hydroxide adjuvant. A third vaccine was prepared utilizing the oligomers (unassembled pilin) in aluminum hydroxide adjuvant.

Groups of two New Zealand white rabbits were immunized with a pilus preparation in a Freund's adjuvant or in aluminum hydroxide adjuvant, or with the unassembled pilin preparation in aluminum hydroxide adjuvant. The immunization schedule was three injections 2 weeks apart followed by bleeding the rabbits one week after the final injection. The rabbits receiving the pili in Freund's adjuvant had the first injection formulated in complete Freund's adjuvant followed by 2 injections in incomplete Freund's adjuvant. These were prepared as an emulsion of a 1:1 mixture (v:v) of antigen to adjuvant, and administered subcutaneously. The pili and unassembled pilin were absorbed with a final concentration of 1.2% aluminum hydroxide and were administered intramuscularly. The amount of material injected was 50 micrograms protein per 0.45kg at each injection. Sera were collected 1 week after the last injection and kept frozen at -40 C until needed.

The titers of the various antisera against purified intact pili were determined by an enzyme linked immunosorbent assay.

As a result of these tests, it was confirmed that the vaccines prepared utilizing unassembled pilin elicited a higher antibody titer against heterologous pili than that elicited by the intact pilus vaccines.

EXAMPLE II

Moraxella bovis isolates FLA64(6), MED72(23R), and EPP63 were obtained from the National Animal Disease Center, AMES, Iowa. M. bovis isolate IM427 was isolated locally from a Holstein cow in Hillsborough County, Florida. The unassembled pilin form of the antigen was prepared from purified pili substantially as described in Example I, above. The pili were converted to unassembled pilin by dialysis for 48 hours against 0.15M phosphate buffer at pH 12.5 at 4 C followed by dialysis against a 0.01 M-Tris (hydroxymethyl)-aminomethane buffer, pH 7.0.

Utilizing the resulting unassembled pilin, vaccines were prepared having four different dosages (50 micrograms, 10 micrograms, 2 micrograms and 0 micrograms) of each antigen. All vaccine formulations included 1.2% aluminum hydroxide as adjuvant.

Sixteen New Zealand white rabbits were immunized, 1 rabbit for each of the different dosage combinations. The rabbits were approximately 8 weeks of age and weighed approximately 1.4 Kg. The final volume of each vaccine formulation was 1 milliliter. All formulations were administered twice intramuscularly in the rear thighs, 30 days apart. Sera were collected weekly. The last bleed was 49 days after the initial injection. Sera was stored at -40 C until needed.

As in Example I, end point titers were determined by an enzyme linked immunosorbent assay.

The administered vaccines were prepared from the unassembled pilin of th FLA64 and MED72 isolates, mixed at various concentrations, adsorbed with an aluminum hydroxide adjuvant. The titers were determined against the two pilus antigens from which the immunogens were prepared, and against pili from two heterologous isolates (EPP63 and IM427).

Test results show that the addition of the FLA64 unassembled pilin to the MED72 unassembled pilin greatly enhanced the antibody response to the MED72 pili. However, the addition of MED72 unassembled pilin to the FLA64 unassembled pilin repressed the antibody response toward the FLA64 pili. As a result, it has been determined that an infectious bovine keratoconjunctivitis (IBK) vaccine can be formulated utilizing unassembled pilin from M. bovis isolates FLA64 and MED72.

In light of these results with regard to unassembled pilin from M. bovis, similar results may be expected with vaccines prepared from unassembled pilin of other bacteria also belonging to the NMePhe pilus group as described above. In similar fashion, this work and applied theory indicates that effective vaccines may also be prepared utilizing unassembled pilin from bacteria that are incapable of completing the assembly of the pilus. Thus, dissociation of the pilus antigens, as set forth in Example I, would not be necessary if unassembled pilin were obtained directly from the bacteria. It is known that a few N. gonorrhoeae isolates synthesize unassembled pili. It is also known, as discussed above, that subinhibitory concentrations of penicillin allow synthesis of the pilin to proceed, but prevents its assembly into pili. Such procedures may, then, also provide unassembled pilin for use in formulating the vaccine of this invention.

It is also to be noted that the unassembled pilin may be lyophylized after collection for subsequent formulation into vaccine preparations. Lyophlization of

the antigen alone, or in combination with pres rvatives may be utilized. It is also to be understood that the vaccine of this invention may be prepared without the use of any adjuvant, but greater concentrations of antigen would have to be administered. In addition to the aluminum hydroxide adjuvant, other proposed adjuvants include alum, aluminum phosphate, emulsified paraffin, emulsified paraffin with lecithin, purified saponin, oils, liposomes, detergent-type micelles, ISCOM's and immunostimulants.

Preservatives such as those having USDA approval may be added to the vaccine formulation, and stabilizers such as boric acid, EDTA, bovine serum albumin, and saccharides may also be utilized.

EXAMPLE III

The following test was conducted to investigate the efficacy of both the whole pilus and pilus oligomer isolated from Moraxella bovis as vaccines against IBK. The vaccines were prepared from the same bacterial strain that was used as the challenge pathogen. This type of experiment is referred to as a homologous challenge experiment.

Eight calves were divided into three groups. The first group of three were vaccinated with the whole pilus preparation. The second group of three were vaccinated with the pilus oligomer preparation. The third group of two received no vaccine and served as controls. The vaccinated calves received two injections of the respective vaccines thirty days apart. Sera from all calves were collected periodically in order to follow anti-pilus antibody levels. Twenty days after the second vaccination, all calves were challenged after stressing their eyes with UV irradiation. Protection against IBK was monitored for 5 days after challenge by examination

of the eyes for gross symptoms and by the recovery of beta hemolytic bacteria from the animals' eyes.

The vaccines were prepared from the EPP63 strain of M. bovis. The intact pili were prepared by scraping the 24 hr. growth from Mueller-Hinton agar into 10 mM, pH 7.0 Tris-HCl buffer. The pili were separated from the bacteria by blending 2 min. and then centrifuging for 20 min. at 20,000 RPM. The supernatants were saved and ammonium sulfate was added to 20% saturation. After incubating 1 to 2 hr., this solution was centrifuged at 18,000 RPM for 2 hr. The pellet was retained and resuspended in the Tris-HCl buffer. After resuspension, usually overnight, the solution was centrifuged at 18,000 RPM for 2 hr. and the supernatant containing the pili was saved. Purity of the preparation was determined by sodium dodecylsulfate-polyacrylamide gel electrophoresis. If the purity was unacceptable, the preparation was recycled again beginning at the ammonium sulfate addition step.

The oligomer was prepared from the purified pili by ultrasonication with a Branson W-350 apparatus using an 18 mm probe for 1 min. at a setting of 10. To complete the conversion to oligomers, the sonicated pili were dialyzed against distilled water adjusted to pH 11.5 with NaOH for 48 to 72 hr. The formation of oligomers was confirmed by "native" polyacrylamide gel electrophoresis and electron microscopy.

The vaccines were prepared by mixing the pili or oligomer for 1 hr. with Rehsorptar II (Armour Pharmaceuticals), an alum based adjuvant. The mixing ratio was 2 parts pili or oligomer to 3 parts Rehsorptar II. A total of 5 mg protein for the whole pilus and a total of 3.5 mg protein of the oligomer were administered intramuscularly (i.m.) per calf in a volume of 8 to 8.4 ml at each inoculation.

In order to achieve infection by M. bovis, the calves' eyes were prestressed using UV light. This was

done to all the calves 48 hr., 24 hr., and just prior to the instillation of the bacteria. The UV stressing was performed by placing a "fresh" G.E. sunlamp (Model No. RSM, 275w, 110-125v) 50 cm from the calf's eye and turning it on for 20 min. The bacterial strain EPP63, a known virulent strain and the same strain from which the vaccines were prepared, was scraped from confluent growth on Mueller-Hinton agar plates which had been incubated for 24 hr. The suspension medium was 10 ml of NIH thioglycollate medium containing 7% $MgCl_2.6H_2O$. Nine tenths of a milliliter were instilled per eye. The number of bacteria used for the challenge was not determined because of the tendency of these bacteria to auto-aggregate.

All eyes were swabbed the day prior to, just prior to, and each day for five days, after challenge. Marion Scientific Culturettes were used for the swabbing and transport of the cultures to the laboratory. The swabs were then promptly streaked onto 5% blood agar plates and incubated overnight at 35 C. Small beta-hemolytic colonies were scored as being M. bovis. No further effort was made to identify the bacteria even though hemolytic activity is known to be associated with other bovine eye flora.

None of the calves receiving either vaccine developed symptoms of IBK, while both control calves developed severe infections. The protective effect of the vaccines was also evident from the relative inability to culture small beta-hemolytic colonies from the challenged animals as compared to the controls. Complete protection by both vaccines appears to have occurred.

Both vaccines elicited increased antibody titers as detected by the ELISA based on the whole pilus as the assay antigen. After the first injection, the titer rose to a plateau on about day 12. The second injection, on day 30, increased the titer even more with the maximum occurring on day 40. Both vaccines induced nearly equal

titers after th first injecti n, but after the second injection, the whole pilus induced the highest titers. This difference in antibody induction, however, may be due to the use of only the whole pilus and not the oligomer as the antigen in the ELISA. The control animals exhibited no increase in antibody titers until after the bacterial challenge. Then a minimal increase in anti-pilus antibody was detected.

In summary, both the whole pilus and oligomer vaccines gave complete protection from the challenge of the homologous strain of M. bovis (EPP63). Initially the oligomer vaccine gave antibody titers comparable to the intact pilus vaccine, as measured by ELISA against whole pili as the antigen. After two injections, the whole pilus vaccine gave the highest antibody titers.

EXAMPLE IV

Having previously demonstrated the efficacy of whole pilus and pilus oligomer vaccines prepared from Morexella bovis in the homologous challenge of Example III, an additional study was conducted to determine whether an oligomer vaccine prepared from the pili of one strain of M. bovis would provide protection against challenge with a strain having serologically different pili. This type of experiment is generally referred to as a heterologous challenge experiment, and the oligomer was prepared from the EPP63 strain and the heterologous challenge strain was FLA64.

Twelve calves were used in the experiment. Nine of the calves were vaccinated with an oligomer preparation made from the EPP63 strain of M. bovis with each calf receiving two injections 30 days apart. The remaining group of three calves was not vaccinated and served as controls. Sera were collected periodically from all calves in order to monitor antibody levels. Sixteen days after the last vaccination, all the calves' eyes were

prestress d using UV light and challenged using two different strains of M. bovis. The first group of three vaccinated calves was challenged with strain EPP63. The second group of three vaccinated calves was challenged with the strain FLA64. The third group of three vaccinated calves was challenged with a mixture of the two strains. One of the controls was challenged with the FLA64 strain, and the remaining two control calves were challenged with the strain mixture. Protection against IBK was monitored for five days after challenge by examination of the eyes for gross clinical signs, and for four days by culturing the animals' eyes for M. bovis.

The oligomer vaccine was prepared from pili purified from strain EPP63 according to the procedure set forth in Example III, above. However, in this example, the pili were broken from the bacteria by blending for two minutes and then centrifuging for 20 minutes at 10,000 RPM. The final concentration of the vaccine preparation was 0.4 mg/ml.

As described above, nine holstein calves received intramuscular injections of the vaccine. A total of 8.4 ml of the oligomer vaccine was injected into the left and right hip of each calf (4.2 ml per site). The total dose per injection per calf was 3.4 mg protein. Three calves received no injections and served as controls. The first injection was given on day one and the second injection on day 30.

In order to achieve infection, the calves' eyes were stressed using UV light as in Example III, but for ten minutes. The schedule for this treatment was 48 hours, 24 hours, and immediately prior to challenge, and 24 hours and 48 hours after challenge. The bacterial challenge material was prepared as set forth in Example III, and the challenge dose was 1 ml of the bacterial suspension per eye instilled on day 45.

The eyes of each calf were examined for clinical signs of infection the day prior to challenge, the day of

challenge, and for four days after challenge. On the fifth day after examination, all the calves were treated with antibiotic (Panalog). All eyes were swabbed the day before, just prior to, and each day for four days after challenge. Marion Scientific Culturettes were used for the swabbing and transport of the cultures to the laboratory.

The oligomer vaccine prepared from EPP63 gave protection against homologous (EPP63) challenge with the clinical signs resolving by day four. This confirmed the previous finding of protection against the homologous strains as set forth in Example III. The vaccine showed protection against the heterologous FLA64 challenge, as well as the mixed homologous and heterologous challenge. The clinical signs for three of six of the vaccinated calves were completely resolved by day five, and the remainder of these calves developed less severe signs than the unvaccinated calves. These observations are very promising and indicate complete protection against the homologous strain, and a substantial degree of cross protection against the heterologous strain.

Since the EPP63 oligomer gave protection against the homologous challenge, the protective value of this antigen has been confirmed. The partial protection against the heterologous FLA64 strain was anticipated for two reasons. Prior studies have demonstrated that FLA64 pili are the least cross reactive with sera raised against EPP63 pili. Second, prior studies have also shown that FLA64 induces keratoconjunctivitis to a greater extent than any other strain. Thus, given the low degree of cross-reactivity of EPP63 with FLA64 and the high degree of virulence of the FLA64 strain, the present finding of partial protection by the EPP63 oligomer vaccine against the strain is a very promising result.

In general, the result of this test suggests a proper strategy for designing an M. bovis vaccine. The oligomer

has demonstrated a degree of cross-protection against a serologically different challenge strain and at a lower chall ng dosage might have been completely protective. However, an optimized vaccine should contain oligomers prepared from a few selected strains expressing the most divergent serotypes. The data from this test support this approach and further indicate that the vaccine should include oligomers prepared from both FLA64 and EPP63.

It will thus be seen that the objects set forth above, among those made apparent from the preceding description, are efficiently attained, and since certain changes may be made in carrying out the above method and in formulating the above vaccine without departing from the scope of the invention, it is intended that all matter contained in the above description shall be interpreted as illustrative and not in a limiting sense.

It is also to be understood that the following claims are intended to cover all of the generic and specific features of the invention herein described, and all statements of the scope of the invention which, as a matter of language, might be said to fall therebetween.

Now that the invention has been described,

What Is Claimed Is:

1. A method for obtaining unassembled bacterial pilus subunits suitable for use in preparing a vaccine, said method comprising the steps of:

a. harvesting the bacteria from their growth medium in a buffer solution of substantially neutral pH;

b. preparing from the harvested bacteria a substantially neutral pH solution containing unassembled pilin; and

c. adjusting the concentration of the unassembled pilin-containing material from step b to obtain the unassembled bacterial pilus subunits suitable for use in preparing a vaccine.

2. A method as in claim 1 wherein the bacteria are harvested in a buffer solution at pH 6-8.

3. A method as in claim 2 wherein the bacteria are harvested in a buffer solution at about pH7.

4. A method as in claim 1 wherein the bacteria are selected from the group consisting of Neisseria gonorrhoeae, Neisseria meningitidis, Moraxella bovis, Moraxella nonliquefaciens, M. catarrhalis, Vibrio sp., Pseudomonas aeruginosa, and Bacteriodes nodosus.

5. A method as in claim 1 wherein the preparation of a substantially neutral pH solution containing unassembled pilin comprises the steps of:

b1. subjecting the solution of harvested bacteria to ultrasonication;

b2. dialyzing the sonicated solution against water at about pH 11.5-12.5 for about 24-72 hours; and

b3. adjusting the pH of the dialyzed solution to substantial neutrality.

6. A method as in claim 5 wherein said step of ultrasonication is conducted at a power of about 35-60 watts for about 2-5 minutes.

7. A method as in claim 6 wherein the bacteria are M. bovis, and wherein said step of ultrasonication is conducted at a power of about 35 watts for about 2 minutes.

8. A method as in claim 7 wherein the sonicated solution is dialyzed against water at about pH 11.5 for about 24-48 hours.

9. A method as in claim 7 wherein said step of ultrasonication is conducted at a power of about 60 watts for about 5 minutes.

10. A method as in claim 9 wherein the sonicated solution is dialyzed against water at about pH 12.5 for about 48-72 hours.

11. A method as in claim 1 wherein the preparation of a substantially neutral pH solution containing unassembled pilin comprises the step of selecting a bacterial isolate that secretes unassembled pilin.

12. A method as in claim 11 wherein the bacterial isolates are selected from N. gonorrhoeae.

13. A method as in claim 1 wherein the preparation of a substantially neutral pH solution containing unassembled pilin
comprises the step of subjecting the harvested bacteria to a subinhibitory concentration of antibiotic to allow synthesis of pilin to proceed, but to prevent its assembly into pili.

14. A method as in claim 13 wherein the antibiotic is selected from the class consisting of tetracycline and penicillin.

15. A method as in claim 14 wherein the antibiotic is penicillin.

16. A vaccine comprising unassembled pilin in a normal saline solution.

17. A vaccine as in claim 16 further comprising an adjuvant.

18. A vaccine as in claim 17 wherein the adjuvant is aluminum hydroxide.

19. A vaccine as in claim 17 further comprising a preservative.

20. A vaccine as in claim 19 further comprising a stabilizer.

21. A method for protecting animals against infection comprising administering an effective amount of a vaccine comprising unassembled pilin in a normal saline solution.

22. A method in claim 21 wherein said unassembled pilin are selected from the bacterial group consisting of Neisseria gonorrhoeae, Neisseria meningitidis, Moraxella bovis, Moraxella nonliquefaciens, M. catarrhalis, Vibrio sp., Pseudomonas aeruginosa, and Bacteriodes nodosus.

INTERNATIONAL SEARCH REP RT

International Application No PCT/US90/01592

I. CLASSIFICATION F SUBJECT MATTER (If several classification symbols apply, indicate all) 3

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC (5): A61K 39/02; C12N 13/00; C12P 21/00
U.S. CL.: 424/192; 435/173, 71.2

II. FIELDS SEARCHED

Minimum Documentation Searched 4

Classification System	Classification Symbols
U.S.	424/92, 89; 435/71.2, 173, 253.1, 253.2, 253.4, 252.1

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 5

III. DOCUMENTS CONSIDERED TO BE RELEVANT 14

Category *	Citation of Document, 16 with indication, where appropriate, of the relevant passages 17	Relevant to Claim No. 18
X	US, A, 4,702,911 (McMICHAEL) 27 October 1987, See columns 1-4.	1-4
Y	US, A, 4,461,838 (BRINTON ET AL) 24 July 1984, See columns 2, (line 9-10), 10, 19, 23, 26	1-12, 17, 19
P,Y	US, A, 4,857,318 (LEE) 15 August 1989, See columns 3-5	16-22
Y	Journal of Bacteriology, volume 163, issued July 1985, C. F. Marrs et al., "Cloning and sequencing of a Moraxella bovis pilin gene", See pages 132 and 137	4, 12, 22
Y	Canadian Journal of Microbiology, volume 29, issued 1983, I. E. Salit, "Effect of subinhibitory concentrations of antimicrobials on Meningococcal Adherence", See pages 369 and 374.	13-15

* Special categories of cited documents: 15

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

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IV. CERTIFICATION

Date of the Actual Completion of the International Search 2	Date of Mailing of this International Search Report 2
05 JUNE 1990	19 JUL 1990
International Searching Authority 1	Signature of Authorized Officer 20
ISA/US	for KAY K. KIM

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